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Human Antibody Response to Thioredoxin Peroxidase-1 and Tandem Repeat Proteins as Immunodiagnostic Antigen Candidates for *Schistosoma japonicum* Infection

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Abstract. Schistosomiasis continues to be a public health problem in many tropical and subtropical countries. Improving the diagnostic tools for surveillance and monitoring in areas that have reached elimination level will help hasten the possible elimination of this disease. This study therefore aims to develop enzyme-linked immunosorbent assay through the use of recombinant proteins such as thioredoxin peroxidase-1 (SjTPx-1) and four tandem repeat proteins (Sj1TR, Sj2TR, Sj4TR, and Sj7TR). Cutoff values were calculated using 38 serum samples from healthy Japanese volunteers. Sera from 35 schistosomiasis-confirmed patients, four cured from the disease by chemotherapy, and 15 endemic negative controls were used to assess these antigens. SjTPx-1 and Sj7TR both had 85.71% sensitivity. Furthermore, these antigens were also tested against human sera positive for other parasitic infections and showed no or very minimal cross-reaction. These results suggest the potential defined antigens for development of an accurate diagnostic test for schistosomiasis.

INTRODUCTION

Since the identification of the *Schistosoma* parasite in Egypt in 1851,[1] schistosomiasis has continued to be a public health problem in 76-endemic countries.[2] More than 207 million people are infected worldwide and about 700 million people are at risk of having the disease.[2] This parasitic disease is far from being eradicated despite national control programs implemented in endemic countries. Improving the diagnostic techniques should be given importance, because schistosomiasis diagnosis is needed for epidemiological studies and evaluating the efficacy of control programs.

Definitive diagnosis of *Schistosoma japonicum* infection in endemic areas still relies on the Kato-Katz technique and/or the sedimentation concentration technique, which are based on the detection of schistosome eggs in the stool of the infected individuals.[3] This technique is simple and has a very high specificity, making it the "gold standard" method for schistosomiasis diagnosis. However, this parasitological technique is labor-intensive, requires skilled personnel, has low sensitivity in low prevalence endemic areas,[4,5] and according to one study, seven repeated Kato-Katz examinations coupled with miracidial hatching is required to have its maximal sensitivity.[6] Because of these issues in sensitivity, crude egg antigen-based immunodiagnostic techniques such as the circum-oval precipitin test (COPT) and enzyme-linked immunosorbent assay (ELISA) are usually used instead. However, it will be difficult to produce crude egg antigen for large-scale diagnostic purposes. And so far, only a few defined antigens have been identified for serological diagnosis of schistosomiasis.[7,8] Therefore, there is a need to develop a recombinant diagnostic antigen that is easier to produce.

The recently completed genome of *S. japonicum*,[9] database available online, made it possible to analyze more antigens to be used possibly in the diagnosis and vaccine development against the parasite. For the diagnostic purposes, producing the recombinant antigens is easier than using the crude egg antigen. This can be an important factor in the success of performing mass diagnosis and epidemiological surveys.

Peroxiredoxin (Prx) is considered as the major detoxifying agent against hydrogen peroxide in helminths,[10] which limits the damage done by this reactive oxygen species.[11] Based on their amino acid sequences, the Prx have been classified into 1-Cys or 2-Cys Prx.[12] The 2-Cys Prx is also known as thioredoxin peroxidase (TPx) because it uses electrons from the thioredoxin system.[10] In *S. japonicum*, TPx exists in three forms, namely TPx-1, TPx-2, and TPx-3.[13] TPx-1 is expressed on the tegument of the adult parasite[14] making it more exposed to the host immune system. Among the three types, only TPx-1 was seen in the excretory/secretory products from the adult worm.[13] In a previous study, it was tested on cattle samples through ELISA and yielded 84.0% sensitivity and 89.0% specificity,[15] whereas the antigen has not been evaluated for human cases. On the other hand, tandem repeat proteins (TRPs) are often targets of humoral responses for protozoan[16] and helminthic parasites.[17] Previous studies showed that computational screening of genomes could identify novel genes encoding TRPs with serological significance from various parasites.[18–20] However, no such proteins have yet been identified as antigens for serodiagnosis of schistosomiasis. In this study, we evaluated the serological efficiency of TPx-1 and four TRPs for the diagnosis of human schistosomiasis as compared with *S. japonicum* soluble egg antigen (SEA) by ELISA.

MATERIALS AND METHODS

Parasite. The Yamanashi strain of *S. japonicum* was maintained using the *Oncomelania nosophora* snails and imprinting control region mice to complete its life cycle.[21] Each mouse was infected with 40 to 60 cercariae by exposing its tail to a cercarial water suspension. After 7 to 8 weeks, the mice were killed to obtain the adult parasite. The adults were collected from the mesenteric veins of the intestine, which were cleaned briefly with normal saline solution. The animal experiments in this study were carried out in compliance with

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the Guide for Animal Experimentation at Dokkyo Medical University Japan.

Human sera. Non-endemic control sera were collected from 38 healthy Japanese volunteers from Tochigi prefecture in May 2003.[22] These subjects were without any risk of contracting *S. japonicum* infection and had no history of traveling to schistosomiasis-endemic areas. Fifteen endemic control sera and four post-treatment samples (1 year after chemotherapy) were collected from Gonzaga, Cagayan, the Philippines. These individuals were confirmed negative through stool examination. The schistosomiasis-positive serum samples were collected from 35 human patients from Leyte, the Philippines.[23] They were diagnosed by the detection of the parasite eggs in their stool. Sera from patients with other parasites, including *Trichuris trichiura* (N = 1), *Plasmodium falciparum* (N = 4), *Plasmodium vivax* (N = 1), and *Entamoeba histolytica* (N = 4) were collected from a schistosomiasis-free area in the Philippines. They were diagnosed through either microscopic examination or detection of antibodies by immunofluorescent assay. *Paragonimus westermani*-positive samples (N = 11) were taken from Japanese patients and *Opisthorchis viverrini*-positive sera (N = 10) were collected from Thailand diagnosed through either clinical manifestations or antibody detection. Blood samples were taken from these subjects after informed consent in their local language were obtained by a medical staff member from each patient or their guardians. This study was done according to the ethical guidelines for epidemiological studies provided by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan. The University of the Philippines, Manila also issued favorable technical and ethical reviews of this study.

Computational search and synthesis of tandem repeats (TR) genes. DNA sequence data from *S. japonicum* (*Sjaponicum_*Genes.v3) were obtained from GeneDB.[24] Tandem Repeats Finder, a program to locate and display TR in DNA sequences,[25] was used to identify TR genes as previously described.[18] In this study, the genes were regarded as TR genes if the scores obtained from the Tandem Repeats Finder analysis were 500 or higher. The biochemical properties of each of the top 20 *S. japonicum* TR genes/proteins were analyzed for 1) a protein's molecular mass, isoelectric point, presence of a signal sequence, trans-membrane domain(s), or a GPI-anchor signal; 2) known antigenicity and/or functions by Blast searches against the National Center for Biotechnology Information (NCBI) database using both DNA and deduced amino acid sequences; 3) evidence of protein expression by comparison with a previous study[26]; 4) transcriptomic expression levels by comparison with the NCBI EST database. On the basis of these analyses, four TR genes were selected for production of recombinant proteins. Nucleotides coding a partial TR domain of the chosen TRPs were synthesized by GenScript USA Inc. (Piscataway, NJ).

Cloning and sequencing of TPx-1. Total RNA was extracted from *S. japonicum* adult worms using TRIzol (Invitrogen, Madison, WI). First strand synthesis of complementary DNA (cDNA) was done using the Ready-To-Go T-Primed First Strand Kit (Amersham Biosciences, UK) and oligo (dT) primer. From the cDNA of the adult worm, SjTPx-1 was amplified by polymerase chain reaction (PCR) using the primer set 5′-TTA GGA TCC ATG GTA CTG ATT CCA AAT-3′ and 5′-TTA AAG CTT TAA TCA GTG ATT CAC TTT-3′ (*Bam*HI and *Hind*III sites were underlined) based on the sequence obtained from GeneDB[27] (accession no. Sjp_0095720.1). The expected length of the PCR product was 555 bp. Twenty microliters of reaction mixture contained 2 μL of buffer, 0.6 μL of 1.5 mM $MgCl_2$, 1.6 μL of 2.5 mM dNTP, 0.4 μL of each 20 pmol/μL primer, 0.2 μL of 5 U/μL *Taq* DNA polymerase (Takara, Otsu, Japan), and 1 μL of template. The conditions for PCR were as follows: 94°C for 5 min, followed by 35 cycles of 30 sec in 94°C, 45 sec in 60°C, and 45 sec in 72°C, and a final extension of 72°C for 10 min. The PCR was performed using Veriti 96-Well Thermal Cycler (Applied Biosystems, Carlsbad, CA). The PCR products were separated by electrophoresis in 1.5% agarose gel and visualized by ethidium bromide staining. The amplified DNA sequence was cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). *Escherichia coli* DH5α were transformed with the plasmid. Selected clones were sequenced to verify the identity of the cloned sequences using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Recombinant protein preparation. The SjTPx-1 gene was digested with the restriction enzymes *Bam*HI and *Hind*III, whereas the four synthesized TRP genes with *Nde*I and *Eco*RI (the enzyme sites added at 5′ and 3′, respectively). The resulting digested genes were inserted into the pET28 vector (EMD Biosciences, San Diego, CA). *Escherichia coli* BL 21 was transfected with pET28 plasmids containing the individual genes and were grown in LB medium (Sigma-Aldrich, St. Louis, MO) supplemented with 50 μg/mL of kanamycin for cloning. The expression of the recombinant proteins in SOB medium (BD, Sparks, MD) was induced with 0.5 mM isopropyl-thio-β-D-galactoside (IPTG) and maintained for 3 h. The recombinant proteins were recovered using the Ni-NTA agarose (Qiagen Inc., Valencia, CA) according to the manufacturer's instruction. SjTPx-1, Sj1TR, Sj2TR, and Sj7TR were purified as soluble proteins, whereas Sj4TR as an insoluble protein. The proteins were eluted and dialyzed with 20 mM Tris, pH 8.0. The integrity and purity of the proteins were evaluated with 15% polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and subsequent Coomassie Brilliant Blue staining (MP Biomedicals, Solon, OH). The concentration of each expressed protein was measured using the BCA Protein Assay (Thermo Scientific, Rockford, IL).

ELISA. A conventional ELISA was done as previously described[28] with slight modifications. In this study, horseradish peroxidase (HRP)-conjugated anti-human immunoglobulin G (IgG) goat serum (Rockland Inc., Gilbertsville, PA) was used for the secondary antibody and 3,3′,5,5′-tetramethylbenzidine (KPL, Gaithersburg, MD) was used as the substrate for HRP. The wells of the microplates (Nunc Maxisorp, Thermo Fisher, Rockland, IL) were sensitized separately with SEA (1 μg/well) or each of the recombinant proteins (200 ng/well). Proteins were diluted with carbonate/bicarbonate buffer at pH 9.6. After blocking with 1% bovine serum albumin (BSA) in phosphate buffered saline with 0.05% Tween 20 (T-PBS) (T-PBS-0.1%BSA), the antigen-coated well was filled with the serum. The test sera (0.1 mL) were diluted 1:400 with T-PBS-0.1%BSA and while the secondary antibody (0.1 mL) was diluted in 1:10,000. Optical density (OD) was measured at 450 nm using a microplate reader (MTP-500, Corona Electric, Tokyo, Japan). All the tests were done in triplicates.

Statistical analysis. The validity of the antigens was estimated by the sensitivity, specificity, and predictive values using the

stool analysis as the reference standard. The agreement between the antigens was estimated by the kappa value.[29]

RESULTS

TR gene analysis. A total of 12,657 gene sequences were analyzed by Tandem Repeats Finder, of which 134 genes were found to have TR regions based on the arbitrary cutoff score of 500. Only eight of them had a score higher than 2,000, which is very few as compared with previous studies on other parasites such as *Leishmania infantum*,[30] *Trypanosoma brucei*,[30] and *P. falciparum*.[31] The top 20 TR genes with the highest scores are shown in Table 1, and four TR genes were selected for production of recombinant proteins based on conservation in other organisms and expression evidence. For example, ubiquitin (Sjp_0031660 and Sjp_0066050) and splicing factor 3G subunit 4 (Sjp_0031090) were excluded from further study because of the high conservation. Although Sjp_0059850 (Sj4TR) showed some similarity to proteins from other organisms, those included higher animals but not pathogens causing diseases to mammalian hosts. Furthermore, genes without expression evidence based on previous proteomic and transcriptomic studies were avoided for further study because they were more likely to be just putative genes.

Cloning and expression of proteins. The PCR amplified SjTPx-1 gene was ~555 bp (Figure 1A) similar to the size reported in the database (GeneDB). The gene showed 98% identity with *S. japonicum* TPx-1 gene (Sjp-0095720.1) in both the nucleotides and amino acid sequences. The gene was conserved in other parasites, with 82% identity to *Schistosoma mansoni* TPx-1 and 61–68% identity to non-schistosome parasites. Understandably, it showed a high percent identity with *S. mansoni*, but the parasitic diseases, which should be considered for possible cross-reaction, are those that can also be seen in the schistosomiasis-endemic areas. In Southeast Asia, schistosomiasis shares geographical endemicity with *Plasmodium* spp. (63% identity), *O. viverrini* (63% identity) and *E. histolytica* (66% identity).

The SDS/PAGE showed that the recombinant proteins in expected size (SjTPx-1, 20 kD; Sj1TR, 19 kD; Sj2TR, 19 kD; Sj4TR, 21 kD, and Sj7TR, 13 kD) were expressed and purified as a single band (Figure 1C–G).

ELISA. The ELISA was performed using sera from 38 Japanese controls, 15 Filipino endemic negative controls, 4 post-treated negative samples, and 35 stool-confirmed schistosomiasis japonica patients. To check their cross-reactivity, sera from 11 *P. westermani*, 10 *O. viverrini*, 1 *T. trichiura*, 3 *P. falciparum*, 1 *P. vivax*, and 4 *E. histolytica* positive patients were included for the ELISA. The cutoff OD value was calculated from the values of the 38 Japanese controls as mean + 3 SD. Thirty-four of the 35 schistosomiasis-confirmed sera were positive for SEA (Figure 2). Eleven of the 15 endemic negative controls were positive for SEA, whereas none for the recombinant antigens. For the post-treatment samples, all were positive for SEA and only one for Sj4TR. SjTPx-1 and Sj7TR both had 30 samples positive of the 35, having 85.71% sensitivity. Sj1TR had 24 positive samples (68.57%) and Sj4TR with 20 positive samples (57.14%). Only three samples were positive for Sj2TR making it not a good candidate for human schistosomiasis diagnosis. For the *P. westermani*, *Plasmodium* spp., and *E. histolytica*-positive samples, results showed no cross-reaction with the recombinant antigens. In contrast, 3 of the 11 *P. westermani*, 5 of the 10 *O. viverrini*, and 2 of the 4 *E. histolytica*-positive samples show high OD values for SEA. Among the recombinant proteins, only 3 of the *O. viverrini*-positive samples showed a very minimal reaction with SjTPx-1.

To test for reproducibility, five independent assays for each recombinant protein using one non-endemic control and one stool-positive control were done on three different plates at the same time. Intra-assay coefficient of variation was below 4% for all the tests (data not shown).

Based on the statistical analysis, SjTPx-1 and Sj7TR showed high agreement with the stool analysis done on the samples based on the kappa values (Table 2). The specificity and the positive predictive values of the four recombinant antigens (SjTPx-1, Sj1TR, Sj4TR, and Sj7TR) were higher than those of SEA.

TABLE 1
Top 20 tandem repeats (TR) genes of *Schistosoma japonicum*

	Gene ID	Product	Score	Identity*	Proteome†	EST	
1	Sjp_0099630	Expressed protein	5085	No	A	yes	Sj1TR
2	Sjp_0086200	Cytoplasmic dynein 1 light intermediate chain 1	3558	No	no	yes	Sj2TR
3	Sjp_0047310	SRP40, C-terminal, domain-containing	2522	No	na	no	
4	Sjp_0002410	Expressed protein	2454	No	na	no	
5	Sjp_0070530	Expressed protein	2357	No	na	no	
6	Sjp_0002010	Melanoma inhibitory activity protein 3 precursor	2189	No	na	no	
7	Sjp_0031660	Ubiquitin C	2154	100%	CSAEM	yes	
8	Sjp_0023440	Mediator of DNA damage checkpoint protein 1	2024	No	na	no	
9	Sjp_0088690	Neuroblast differentiation-associated protein AHNAK	1978	No	na	no	
10	Sjp_0066050	Ubiquitin B	1763	100%	na	yes	
11	Sjp_0012130	Thrombospondin type 3 repeat	1758	42%	na	yes	
12	Sjp_0059850	1-phosphatidylinositol-4-phosphate 5-kinase	1743	53%	C	yes	Sj4TR
13	Sjp_0020150	Kringle-like fold, domain-containing	1715	no	na	no	
14	Sjp_0008730	Polypeptide N-acetylgalactosaminyltransferase	1570	54%	CSM	yes	
15	Sjp_0031090	Splicing factor 3B subunit 4	1562	93%	no	yes	
16	Sjp_0087830	Conserved hypothetical protein	1536	no	no	yes	
17	Sjp_0089830	HMG-I and HMG-Y, DNA-binding	1463	no	na	no	
18	Sjp_0030930	Erythrocyte band 7 integral membrane protein	1457	50%	na	no	
19	Sjp_0110390	Expressed protein	1391	no	no	yes	Sj7TR
20	Sjp_0069600	Protein kinase PKN/PRK1, effector, domain-containing	1389	no	CM	yes	

*The highest percent identity of the repeat motif to proteins from organisms other than *Schistosoma* species. "no" indicates that the motif was not found in the other organisms.
†Protein expression evidence was based on Liu and others.[26] C = cercariae; S = hepatic schistosomula; A = adults; E = eggs; M = miracidia; No = not detectable; na = data not available.

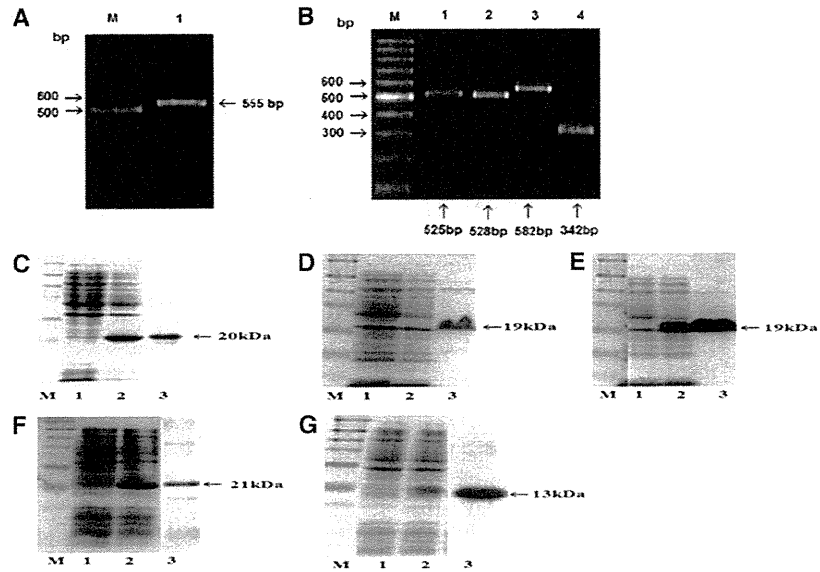

FIGURE 1. Gel electrophoresis and polyacrylamide gel electrophoresis (SDS-PAGE) of the recombinant antigens. (**A** and **B**) The recombinant plasmids were identified by polymerase chain reaction (PCR) and enzyme digestion. (**A**) Lane 1: TPx-1 from the *Schistosoma japonicum* adult worm cDNA library. (**B**) Synthesized TRP genes. Lane 1: Sj1TR; Lane 2: Sj2TR; Lane 3: Sj4TR; Lane 4, Sj7TR. M = Marker. (**C–G**) Expression and purification of the recombinant proteins. M = Marker. Lane 1: *Escherichia coli* culture before adding isopropyl-thio-β-D-galactoside (IPTG) and Lane 2: after adding IPTG. (**C**) SjTPx-1. (**D**) Sj1TR. (**E**) Sj2TR. (**F**) Sj4TR. (**G**) Sj7TR.

DISCUSSION

To date, there are only a few defined antigens evaluated for diagnostic purposes in schistosomiasis, most of which were only tested against animal sera.[15,32–34] Previous results showed high sensitivity and specificity of the candidate antigens but further study is needed to know their true immunodiagnostic potential on human schistosomiasis. Therefore, other antigens should still be tested and examined for comparative evaluation with those already used. In this study, we assessed the

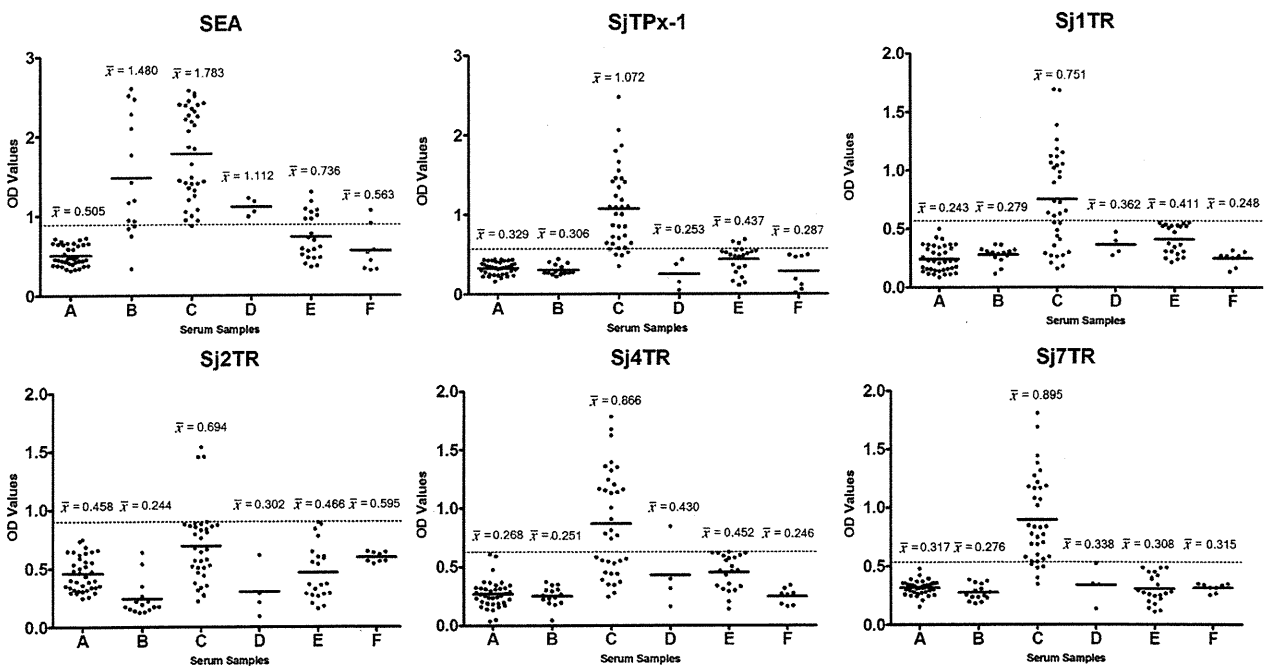

FIGURE 2. Enzyme-linked immunosorbent assay (ELISA) results of soluble egg antigen (SEA) and the recombinant antigens. (**A**) Negative serum samples from Japanese volunteers. (**B**) Negative endemic controls from Filipino volunteers. (**C**) *Schistosoma japonicum* stool-positive serum samples. (**D**) Post-treatment negative serum samples. (**E**) Sera positive for other heminthic infection (*Paragonimus westermani*, $N = 11$; *Opisthorchis viverrini*, $N = 10$; *Trichuris trichiura*, $N = 1$). (**F**) Sera positive for protozoan infection (*Plasmodium falciparum*, $N = 3$; *Plasmodium vivax*, $N = 1$; *Entamoeba histolytica*, $N = 4$). Mean optical density (OD) values (x) are given on each category of samples.

TABLE 2
Statistical analysis of the ELISA results of SEA and the recombinant proteins*

Antigen	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa†
SEA	97.14	71.76	58.62	98.38	0.577
SjTPx-1	85.71	96.47	90.91	94.25	0.836
Sj1TR	68.57	100	100	88.54	0.756
Sj4TR	57.14	98.82	95.24	84.85	0.634
Sj7TR	85.71	100	100	94.44	0.895

*ELISA = enzyme-linked immunosorbent assay; SEA = soluble egg antigen; PPV = positive predictive values; NPV = negative predictive value.
†Kappa values of SjTPx-1 and Sj7TR showed high agreement with the stool analysis based on the previously reported criteria set.[29]

immunodiagnostic potential of SjTPx-1 and four TR proteins using human samples.

The recombinant SjTPx-1 and Sj7TR both showed an 85.71% positivity rate on the schistosomiasis-confirmed human samples as compared with the 97.14% positivity rate of SEA. Of the 35 stool-positive samples, 32 were positive for either one or both of the SjTPx-1 and Sj7TR; therefore, complementing these two antigens, there will be a possible maximal yield of 91.43% sensitivity. Fusion proteins have been shown effective in the serodiagnosis of other parasites.[35,36] On the basis of the ELISA results, Sj7TR is a promising candidate antigen for diagnostic purposes, although GeneDB defined it only as an expressed protein. Immunolocalization and expression profiling therefore of this schistosome antigen should be done to understand its role for the parasite.

The sensitivity of SjTPx-1 using human samples was comparable to that of water buffaloes in a previous study.[15] However, the recombinant TRPs should also be tested for their applicability to other animal reservoirs. Sj1TR, which detected lower antibodies in human schistosomiasis patients than Sj7TR, showed higher reactivity to sera from water buffaloes with suspected infection of *S. japonicum* (Angeles and others, unpublished data). Such variation in antigenicity between different hosts has been found in other parasitic diseases.[37] Differences in immune responses to *S. japonicum* antigens between humans and other animal hosts may be derived from different survival mechanisms in these hosts because of their diverse immunological backgrounds depending mostly on the host's immunological memory. This includes the immunodominance of the primary response,[38] which means to which epitopes did the host responds; and to which of the primary epitopes has the host retained its memory. It will be intriguing to further characterize such differentially recognized antigens in terms of expression levels and functions in such hosts. In addition, strain diversity of these recombinant proteins should also be studied in future researches.

The relationship between the intensity of infection using the number of eggs per gram and the antibody titer measured against the recombinant antigens was also studied (data not shown). The result showed no correlation between the two quantitative measures. This might be explained by the idea that antibody production is not merely caused by the number of eggs released by the schistosome parasite, but also to the host's ability to produce antibody against certain epitopes of the antigen.

The use of *S. japonicum* SEA in immunological tests has been known to cause false-positive results with other parasitic and viral diseases.[39] As seen in the results, SEA showed cross-reaction with *P. westermani*, *O. viverrini*, and *E. histolytica*-positive samples. Only SjTPx-1 showed a very minimal cross-reaction with *O. viverrini*-positive sera, whereas none for the recombinant tandem repeat proteins. This proves that recombinant proteins are more specific than the crude antigen.

Mass chemotherapy[40] has been the main strategy in the control of schistosomiasis in high prevalence, whereas selective treatment is used in low prevalence areas. Hence, there is a need for a sensitive diagnostic tool for the cases to be identified and given treatment. In this study, endemic negative controls and samples from persons previously treated for schistosomiasis tested positive only with the crude antigen. This is proof that recombinant proteins can be used to identify true positives in schistosomiasis-endemic areas. The use of the recombinant proteins will therefore be critical in surveillance and monitoring in areas where the prevalence level has reached the elimination level. Furthermore, this may also be applied to epidemiological studies and animal reservoir surveillance of the disease, which need highly specific tests. Hence, the real epidemiological picture of schistosomiasis can be shown, which can further help in the possible elimination of the disease.

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Complete mitochondrial genomes of *Diplogonoporus balaenopterae* and *Diplogonoporus grandis* (Cestoda: Diphyllobothriidae) and clarification of their taxonomic relationships ☆

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ABSTRACT

Although the diplogonadic human tapeworm, *Diplogonoporus grandis*, has long been considered to be a synonym of the whale tapeworm, *Diplogonoporus balaenopterae*, the identity of the both species at the complete mitochondrial genomes and nuclear DNA levels has been not sufficiently undertaken to date. In the present study, to clarify the taxonomic relationships between *D. balaenopterae* and *D. grandis* at the molecular level, the complete mitochondrial genomes of both species were sequenced and compared. In addition, the genetic variation in the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) and the nuclear internal transcribed spacer-1 (ITS-1) region of the ribosomal RNA gene were examined. The complete mitochondrial genomes of *D. balaenopterae* and *D. grandis* consisted of 13,724 bp and 13,725 bp, respectively. These mitochondrial genomes contained 12 protein-coding, 22 transfer RNA and 2 ribosomal RNA genes and two longer non-coding regions. Except for *Hymenolepis diminuta*, the genomic organization in both species was essentially identical to that in other cestode genomes examined to date. However, differences were observed between *Diplogonoporus* and *Diphyllobothrium* species in abbreviated stop codons, sequences and the number of repeat units in the 2nd non-coding regions. The genetic differences observed in the mitochondrial genomes, *cox1* and ITS-1 regions of both species were considered typical of intraspecific variation, implying that *D. grandis* is a valid name and which is a junior synonym of *D. balaenopterae*. Further, molecular-phylogenetic analysis confirmed that *D. balaenopterae* is more closely related to *Diphyllobothrium stemmacephalum*, the type-species of the genus *Diphyllobothrium*, and the taxonomical validity of the genera *Diplogonoporus* and *Diphyllobothrium* was also discussed.

1. Introduction

Broad tapeworms belonging to the genus *Diplogonoporus* Lönnberg, 1892, are characterized by having a double set of genitalia in a single proglottid, distinguishing them from *Tetragonoporus* Skriabin, 1961, *Hexagonoporus* Gubanov in Delyamure, 1955, and *Polygonoporus* Skriabin, 1967, which all possess multiple gonads in a single proglottid, and many members of *Diphyllobothrium* Cobbold, 1858, which usually have only one set of gonads per proglottid [1]. *Diplogonoporus balaenopterae* Lönnberg, 1892, infects the small intestine of whales, such as the minke whale (*Balaenoptera acutorostrata*, Balaenopteridae, Cetacea), sei whale (*Balaenoptera borealis*, Balaenopteridae, Cetacea) and humpback whale (*Megaptera novaeangliae*, Balaenopteridae, Cetacea) [1]. In contrast, *Diplogonoporus grandis* Lühe, 1899, which causes diplogonoporiasis in humans, has been regarded as a synonym of *D. balaenopterae*, based on adult tapeworm morphology [2–5], characteristics of larval coracidia and procercoids [6,7], and protein profiles [8]. Nevertheless, *D. grandis* has been referred to as the causative agent of human diplogonoporiasis, especially in Japan [9,10].

Diplogonoporiasis cases in humans have been found almost exclusively in Japan where more than 200 cases have been reported over the last 100 years [11]. Outside Japan, a total of 3 cases have been reported in Chile [12], Korea [13] and Spain [14]. In the latter two cases, the etiologic agents were identified as *D. balaenopterae* based on proglottid morphology. While the complete life cycles of these tapeworms have not yet been elucidated, Japanese anchovy or "shirasu" (*Engraulis japonica*, Engraulidae, Clupeiformes), Japanese sardine

Abbreviations: *atp6*, ATPase subunit 6 gene; *cob*, cytochrome *b* gene; *cox1–cox3*, cytochrome *c* oxidase subunits 1–3 genes; *nad1–nad6*, NADH dehydrogenase subunits 1–6 genes; *nad4L*, NADH dehydrogenase 4 large subunit gene; *rnl*, ribosomal RNA large subunit gene; *rns*, ribosomal RNA small subunit gene; *trn*, transfer RNA genes; PCR, polymerase chain reaction.

☆ Nucleotide sequences of the *D. balaenopterae* and *D. grandis* mitochondrial genomes reported in the present paper are deposited at the DDBJ/GenBank databases under accession numbers AB425839 and AB425840, respectively. AB355622–AB355626, AB355628, AB355629, AB474567, and AB474568 are the accession numbers for the *cox1* gene and AB449346–AB449356, AB474569, and AB474570 are the accession numbers for the ITS-1 regions.

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t1.1 **Table 1**
Diplogonoporus isolates examined in the present study.

t1.3 Sample no.	Year collected	Locality collected (latitude/longitude)	Host animals	DDBJ/GenBank accession numbers	
t1.4				*cox1*	ITS-1
t1.5 No. 1	1997	Western North Pacific Ocean (N37/E160)	*Balaenoptera acutorostrata* (minke whale)	AB355622	AB449346
t1.6 No. 2[a]	1997	Western North Pacific Ocean (N37/E163)	*B. acutorostrata*	AB355623	AB449347
t1.7 No. 3	1997	Western North Pacific Ocean (N39/E161)	*B. acutorostrata*	AB355624	AB449342
t1.8 No. 4	1997	Western North Pacific Ocean (N39/E161)	*B. acutorostrata*	AB355625	AB449349–AB449352
t1.9 No. 5	1997	Western North Pacific Ocean (N39/E158)	*B. acutorostrata*	AB355626	AB449353
t1.10 No. 6	2002	Western North Pacific Ocean	*Balaenoptera borealis* (sei whale)	AB474567	AB474569
t1.11 No. 7	2002	Western North Pacific Ocean	*B. borealis*	AB474568	AB474570
t1.12 No. 8[a]	2004	Tokyo, Japan	*Homo sapiens* (58-year-old Japanese man)	AB355628	AB449354
t1.13 No. 9	2006	Hamamatsu, Shizuoka, Japan	*H. sapiens* (58-year-old Japanese man)	AB355629	AB449355, AB499356

t1.14 [a] *Diplogonoporus* isolates used for the complete mitochondrial genome analysis.

(*Sardinops melanostictus*, Clupeidae, Clupeiformes) [9] and skipjack tuna (*Katsuwonus pelamis*, Scombridae, Perciformes) have been suspected to be the most likely sources of infection in humans [10].

To assess the phylogenetic relationships among eucestodes, genes such as the ribosomal RNA large subunit (28S rRNA) and small subunit (12S rRNA) genes [15–20], *cox1* and *nad3*[21], elongation factor-1 alpha gene [16], the internal transcribed spacer (ITS) regions [22,23] and the 18S rRNA gene [19,24] have been used. With regard to *Diplogonoporus* isolates, preliminary DNA analysis using *cox1* recently supported the assignment of *D. grandis* as a synonym of *D. balaenopterae* [25,26]. Genetic analysis of *Diplogonoporus* isolates from clinical cases has recently revealed a close relationship between *Diplogonoporus* and *Diphyllobothrium stemmacephalum*[24].

Thus, in the present study, the complete mitochondrial genomes of both species were sequenced and compared in order to clarify the molecular–taxonomic relationship between *D. balaenopterae* and *D. grandis*. In addition, the genetic variation within the *cox1* and ITS-1 regions was examined using nine *Diplogonoporus* isolates obtained from whales and humans. The need for a revision of the taxonomic affiliation of the genera *Diplogonoporus* and *Diphyllobothrium* is also discussed.

2. Materials and methods

2.1. Diplogonoporus tapeworms examined in the present study

The *Diplogonoporus* tapeworm specimens examined in this study are listed in Table 1. Minke whale and sei whale were taken in the Western North Pacific Ocean with special permission from The Institute of Cetacean Research, Japan. The seven mature *Diplogonoporus* tapeworms collected from the small intestines of the whales were identified as *D. balaenopterae* based on morphological characters (Nos. 1–7 in Table 1). Two diplogonadic tapeworms, one immature tapeworm lacking a scolex (No. 8) and a mature tapeworm with a scolex (No. 9), were obtained from two Japanese patients; these samples were identified morphologically as *D. grandis*. The tapeworms were rinsed thoroughly in phosphate-buffered saline after collection and preserved in 80% ethanol, except for two specimens (Nos. 6 and 9) which were fixed in 10% formalin.

2.2. DNA extraction, PCR amplification and DNA sequencing

DNA was extracted from the ethanol-fixed proglottids using a DNeasy Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. The formalin-fixed specimens were embedded in paraffin and DNA was efficiently extracted from the unstained, 10 μm-thick sections using a DEXPAT kit (Takara Bio Inc., Japan) as described previously [26,27]. For analysis of the complete mitochondrial genome, DNA was extracted from two representative tapeworms fixed in ethanol (Nos. 2 and 8 in Table 1). Amplification of the mitochondrial genomes was performed using 13 primer pairs (Table 2) designed based on the mitochondrial genomes of *Diphyllobothrium nihonkaiense* (AB268585) and *Diphyllobothrium latum* (AB269325). The PCR consisted of an initial denaturation step of 98 °C for 30 s, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s, with a final extension cycle of 72 °C for 5 min. Samples were amplified in a final reaction volume of 50 μL with *Ex Taq* DNA polymerase (Hot Start version, Takara Bio Inc., Japan).

For the polymorphism analysis of the *cox1* and ITS-1 regions of the ethanol-fixed samples (Nos. 1, 3–5 and 7), primer pairs P1/P2 and P28/P31 were designed based on the nucleotide sequences of the *cox1* and ITS-1 regions in *Diplogonoporus* and *Diphyllobothrium*, respectively (Table 3). For the formalin-fixed samples (Nos. 6 and 9), short and overlapping DNA fragments were amplified using primer pairs P3–P27, and P28/P29 and P30/P31 (Table 3) for the *cox1* and the ITS-1 regions, respectively. High fidelity *KOD FX* DNA polymerase (Toyobo, Japan) was occasionally used for DNA samples extracted from formalin-fixed materials. PCR performed using *KOD FX* DNA polymerase employed an initial denaturation step of 94 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s and a final cycle of 72 °C for 5 min.

Amplicons confirmed by agarose gel or capillary electrophoresis (HAD-GT12, eGene Inc., LA) were purified using a NucleoSpin Extract II kit (Macherey-Nagel, Germany) and used as templates for direct DNA sequencing. Samples for DNA sequencing were prepared using

Table 2 t2.1
Oligonucleotide primers used for amplification of mitochondrial genomes.

t2.2 Primer pairs	Nucleotide sequences (5′ to 3′) t2.3
P1 atp6/Fl	ATGATCTTCAGTGGTTATTCAAGTT t2.4
P2 nad1/R25	CACCTGTTAAAAACATAAAAATCAT t2.5
P3 trnA/F	ACAGAATACTGGGTTTGCGTCTCAG t2.6
P4 nad3/R60	AAATGATATGACTATAAACAACJAA t2.7
P5 nad3/F1	ATGTTAGCTTATTTTTTGGTGG t2.8
P6 rnL/R250	CTATACACATTTACITGTCTCCTC t2.9
P7 trnT,F38	CAGGGGTGGGTTTACTCTTGGGCCT t2.10
P8 trnC/R25	TACTAAGACCAAAGGCAATAGACTT t2.11
P9 rnL/F451	CATATTATAAATTTTATATGTAGG t2.12
Pl0 trnC/R25	TACTAAGACCAAGGCAATAGACTT t2.13
P11 rnL/F880	TGAGGTGAGTTAAGACCGGCGTGAG t2.14
P12 rnS/R245	ATTTCACCTACTCTTACCTTTACCT t2.15
P13 trnC/F4O	GTGAATATTGTTTATTCTAGGCTTT t2.16
P14 cox2/R25	CGTAGTACAGCAAAGAAAATTTCAT t2.17
P15 rnS/F570	GTAACAAGGTAGCCCAGATGAATC t2.18
P16 trnE/R25	TTATGCTCCAATACAACAAAACAGG t2.19
P17 cox2/F525	GGTGGGTCACEGTTATATGCCIATA t2.20
P18 nad/R761	CAAGTGGATATGGCAACTATCTTCT t2.21
P19 nad5/F565	CTACCCCTGTTAGTTCTTTAGTACA t2.22
P20 cox3/R205	TACCAAAGGCTAAAACITCIAAG t2.23
P21 trnG/F40	GTGGGGATCTAATGGTTTTAGATAA t2.24
P22 trnH/R25	GCCAGTTTAAATAACCTATCAGTAA t2.25
P23 cox3/F444	GGTTCTAGATTTTATGCTAGTTGT t2.26
P24 nad4/R 325	ACAGAGGTAACATGGATAGCTCATA t2.27
P25 nad4/F1	ATGAGAGTGTACAAAATTATTAGAT t2.28
P26 atp/R25	AATCTTGAATAACCATAAAGATCAT t2.29

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Table 3
Oligonucleotide primers used for the amplification of *cox1* genes and ITS-1 regions.

Primers	Nucleotide sequence (5′ to 3′)
P1 nad3/Fi20	CGAGTGTGGTTTTAGATCTTCTTCA
P2 rnL/R250	CTATACACATTTACTTGATCTCCTC
P3 nad3/F120	CGAGTGTGGTTTTAGATCTTCTTCA
P4 cox1/R125	ATACGTATCATAACACTAAGGCTCA
P5 cox1/F1	ATGATAATCTTAAAGTTTTTAGTT
P6 cox1/R225	GGGCATCAAAAAAAAGAATATCATT
P7 cox1/F101	TGAGCCTTAGTGTTATGATACGTAT
P8 cox1/R325	ATAAAATCMGCATTAMGCTTT
P9 cox1/F201	AATGATATTCTTTTTTTTGATGCCC
Pl0 cox1/R425	TTTCTATCCCTAAAAAGAGCAGAAG
P11 cox1/F301	AAAGCTTTAAGTGCTTGATTGTTAT
P12 cox1/R525	ATCAAAAAAGCTGTGTACAGGGTA
P13 cox1/F501	TACCTGTACACAGCTTTTGTTGAT
P14 cox1/R725	TCAGGATGACCAAAAAATCAAAACA
P15 cox1/F601	GTTTTAGCTGCTGCTATTACMTGT
P16 cox1/R825	AAATAATAMCCATAAAATCCAAAA
P17 cox1/F701	TGTTTTGATTTTTTGGTCATCCTGA
P18 cox1/R925	TAATGACTAAAAAAAAACAGTGT
P19 cox1/F901	ACAGCTGTTTTTTTTAGGTCAGTTA
P20 cox1/R1125	AACCACAAATCAAGTATCATGCTTT
P21 cox1/F1080	TGCTTGTGTTCTTGATAATATTTTG
P22 cox1/R1309	AACCGCATATACCAAAGTAATGCAT
P23 cox1/F1230	ATTGCAGTGTATTGTATAGTGTCT
P24 cox1/R1435	TAACTAGAGACTCCCACAAAATAAAC
P25 cox1/F1401	TTTTTTTGTGTTTATTTTGTGGGAG
P26 cox1/R1566	CTATAAGGCCAACATATATAATCTACAAA
P27 trnT/R23	ACAAAACCAGTATTCTAATTAAA
P28 ITS-1/F1	ACCTGCGGAAGGATCATTACACGTT
P29 ITS-1/R325	ACACGACGCCTCGAGTCTTACGCCT
P30 ITS-1/F301	AGGCGTAAGACTCGAGGCGTCGTGT
P31 ITS-1/R625	AATTCACACAGTTGGCTGCGCTCTTC

an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., CA) and nucleotide sequences were determined by primer walking using an ABI PRISM 3100-*Advant* Genetic Analyzer (Applied Biosystems Inc.).

2.3. Data processing and phylogenetic analysis

A number of models for genetic distance analysis and estimation of phylogenetic trees have been proposed and differ in terms of the parameters used to describe the rates of nucleotide substitution during evolution [28]. In this paper, genetic distance of 36 genes encoded in the mitochondrial genomes between *D. balaenopterae* and *D. grandis*, genetic variation of *cox1* and ITS-1 regions among *Diplogonoporus* isolates was estimated using Kimura's 2-parameter (K2P). The model distinguishes between transitional and transversal substitutions of the nucleotides and assumes that all of the bases are equally frequent [29]. The rate of transitional substitution is often higher than that of transversal substitution, especially in animal mitochondrial DNA, and the model was considered to be suitable for estimating genetic distance of mitochondrial DNA [30]. Genetic distance was calculated by the setting of substitution included transitions and transversions, and the rates among sites (gamma parameter = 1).

Phylogenetic analyses of *Diplogonoporus* and *Diphyllobothrium* were performed using nucleotide sequences of *cox1* from 12 Diphyllobothriidae taxa (DQ768191, AB369249, AB015753, AB517949, EU241311, EU241317, EU241308, FM209182, AB510496, AM412738, AB268585, AB364645), *Bothriocephalus acheilognathi* (Bothriocephalidae, Bothriocephalidea, HM439384) and *Taenia solium* (Taeniidae, Cyclophyllidea, AB516957) were used for comparisons. The nucleotide sequence data were initially aligned with the ClustalW program (ver. 1.83, http://www.clustalw.ddbj.nig.ac.jp). The maximum likelihood analysis (ML) and Bayesian inference (BI) were employed for phylogenetic analyses. For the ML, the alignment data were converted to FASTA format using MEGA program (ver. 5.05) and analyzed using Hasegawa-Kishino-Yano (HKY) + G model (gamma = 5) which was selected based on Bayesian Information Criterion scores using MEGA 5.05.

For BI, the alignment data were converted to NEXUS format using the ClustalX2 (ver. 2.0.12) and Bayesian phylogenetic analysis was performed using MrBayes 3.1.2 (http://mrbayes.csit.fsu.edu/index.php). The DNA data were divided into 3 partitions and likelihood setting was set to nst = 6, equivalent to the general time reversible (GTR) model of nucleotide substitution [31]. Markov Chain Monte Carlo analysis was then run on each of the datasets for 10^6 generations to estimate the posterior probabilities of trees [32]. Phylogenetic trees were rooted with *T. solium* as the outgroup. For the ML analysis, nodal support was assessed by bootstrap resampling (1000 replicates). In BI, clades were considered to have high nodal support if the BI posterior probability was ≥0.95.

3. Results and discussion

3.1. Characterization of the complete mitochondrial genomes of D. balaenopterae and D. grandis

The 5′- and 3′-ends of the protein-coding genes were deduced from the sequences corresponding to the genes in *D. nihonkaiense* and *D. latum*. Using sequence motifs, genes for 2 rRNAs and 22 tRNAs were also annotated in the 5′- and 3′-flanking regions of the open reading frames. In the 2nd non-coding region (NCR2), located between *nad5* and *trnG*, at least three ladder-products were amplified in both species examined (data not shown), with the largest product (320 bp) used to estimate the whole genome size. The complete mitochondrial genome sizes of the isolates No. 2 (*D. balaenopterae*, AB425839) and No. 8 (*D. grandis*, AB425840) were thus estimated to be 13,724 bp and 13,725 bp, respectively.

As shown in Table 4, the mitochondrial genomes consisted of 12 protein-coding genes (*atp6*, *cob*, *cox1–cox3*, *nad1–nad6*), 2 rRNA genes (*rnl*, *rns*), 22 tRNA genes (*trns*), and two longer non-coding regions (NCR1, NCR2). The gene encoding ATPase subunit 8, as well as other flatworm mitochondrial genomes, was absent. Most of the genes were interrupted by several bases and no introns were present in the protein-coding genes. Genes were arranged unidirectionally and exhibited a strong bias toward adenine (A) and thymine (T), with the A + T content reaching 68.7–68.8%. Genomic organization was essentially identical to that reported in other diphyllobothriidean and cyclophyllidean cestodes to date, except for *Hymenolepis diminuta*[33–37].

Of the 12 protein-coding genes, 11 were initiated by an ATG codon, while *cox3* was initiated by GTG (Table 4). Eleven protein-coding genes were predicted to end with complete stop codons: five (*atp6*, *cox2*, *nad2*, *nad5* and *nad6*) were predicted to terminate with TAA and six (*cob*, *cox1*, *nad1*, *nad3*, *nad4* and *nad4L*) with a TAG codon. However, *cox3* in the *Diplogonoporus* isolates was predicted to end with an abbreviated stop codon, T (Table 4), and a complete stop codon appeared to be missing downstream of *trnH*, suggesting that the abbreviated T has been modified by post-transcriptional polyadenylation. It is not considered unusual to find incomplete termination codons, such as T or TA, in the protein-coding genes of metazoan mitochondrial genomes [38]. Indeed, in the mitochondrial genome sequences of flatworms that have been reported to date, abbreviated stop codons have been identified in *cox3*, *nad1* and *nad3* of *D. nihonkaiense* and *D. latum*[34], and *nad1* in *T. solium*[35].

The NCR2 region is considered to be involved in the replication of mitochondrial DNA [39]. Kim et al. [37] reported that size variation in the NCR2 region is largely due to differences in the number of identical 36-nucleotide repeat sequence units, and that these differences vary according to geographic location in *D. latum* (4–6 repeats) and *D. nihonkaiense* (4–7 repeats). In the NCR2 region of *Diplogonoporus* isolates from whales and humans, at least 8 identical repeat units consisting of 33 nucleotides (5′-TTAGGGATGTGTGTAGTATATTCTCTAAATTTG-3′)

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t4.1 **Table 4**
t4.2 Location of genes and non-coding regions (NCR) in the mitochondrial genomes of *D. balaenopterae* and *D. grandis*.

t4.3 Genes and NCRs	*D. balaenopterae* (13,724 bp)			*D. grandis* (13,725 bp)		
t4.4	Locations	Length (bp)	Start + Stop codons	Locations	Length (bp)	Start + Stop codons
t4.5 *trnY*	1–66	66		1–66	66	
t4.6 1st NCR	67–291	225		67–292	226	
t4.7 *trnL1* (CUN)	292–358	67		293–359	67	
t4.8 *trnS2* (UCN)	380–445	66		379–444	66	
t4.9 *trnL2* (UUR)	450–513	64		449–512	64	
t4.10 *trnR*	514–568	55		513–567	55	
t4.11 *nad5*	572–2140	1569	ATG-TAA	571–2139	1569	ATG-TAA
t4.12 2nd NCR	2141–2460	320		2140–2459	320	
t4.13 *trnG*	2461–2528	68		2460–2527	68	
t4.14 *cox3*	2532–3174	643	GTG + T[a]	2531–3173	643	GTG + T[a]
t4.15 *trnH*	3175–3238	64		3174–3237	64	
t4.16 *cob*	3242–4348	1107	ATG-TAG	3241–4347	1107	ATG-TAG
t4.17 *nad4L*	4350–4610	261	ATG-TAG	4349–4609	261	ATG-TAG
t4.18 *nad4*	4571–5821	1251	ATG-TAG	4570–5820	1251	ATG-TAG
t4.19 *trnQ*	5822–5884	63		5821–5883	63	
t4.20 *trnF*	5881–5945	65		5880–5944	65	
t4.21 *trnM*	5942–6007	66		5941–6006	66	
t4.22 *atp6*	6011–6520	510	ATG-TAA	6010–6519	510	ATG-TAA
t4.23 *nad2*	6523–7401	879	ATG-TM	6522–7400	879	ATG-TM
t4.24 *trnV*	7404–7468	65		7403–7467	65	
t4.25 *trnA*	7471–7533	63		7470–7532	63	
t4.26 *trnD*	7537–7598	62		7536–7597	62	
t4.27 *nad1*	7599–8489	891	ATG-TAG	7598–8488	891	ATG-TAG
t4.28 *trnN*	8489–8553	65		8488–8552	65	
t4.29 *trnP*	8567–8629	63		8566–8628	63	
t4.30 *trnI*	8640–8702	63		8639–8701	63	
t4.31 *trnK*	8709–8773	65		8708–8772	65	
t4.32 *nad3*	8775–9131	357	ATG-TAG	8774–9130	357	ATG-TAG
t4.33 *trnS1* (AGN)	9121–9179	59		9120–9178	59	
t4.34 *trnW*	9181–9243	63		9180–9242	63	
t4.35 *cox1*	9252–10,817	1566	ATG-TAG	9251–10,816	1566	ATG-TAG
t4.36 *trnT*	10,808–10,869	62		10,807–10,868	62	
t4.37 *rnl*	10,870–11,831	962		10,869–11,831	963	
t4.38 *trnC*	11,832–11,895	64		11,832–11,895	64	
t4.39 *rns*	11,896–12,625	730		11,896–12,626	731	
t4.40 *cox2*	12,626–13,195	570	ATG-TAA	12,627–13,196	570	ATG-TAA
t4.41 *trnE*	13,198–13,266	69		13,199–13,267	69	
t4.42 *nad6*	13,263–13,721	459	ATG-TAA	13,264–13,722	459	ATG-TAA

t4.43 [a] Abbreviated stop codon.

were confirmed, but variation in the number of repeat units among geographical isolates of *Diplogonoporus* has not yet been thoroughly investigated.

3.2. Taxonomic status between *D. balaenopterae* and *D. grandis* based on molecular analyses

Genetic distances of 36 genes encoded by the mitochondrial genomes of both species were calculated using K2P model. The genetic distance values between the 12 protein-coding genes were extremely small (d = 0.0000–0.0054). Similarly, the genetic distances between the *rnl* and *rns* genes were low at 0.0012 and 0.0030, respectively. Of the 22 *trns*, 19 had identical sequences and *trnC*, *trnH* and *trnV* were separated by a distance of d = 0.0158. By way of comparison, the genetic distance value obtained for the *cox2* in *D. nihonkaiense* and *D. latum*, which are distinct species, was 0.069. Indeed, the value for *cox2* was the lowest genetic distance observed among all of the protein-coding genes of *D. nihonkaiense* and *D. latum* (data not shown), indicating that the differences between *D. balaenopterae* and *D. grandis* were more likely due to intraspecific variation than because the two taxa were separate species.

Fig. 1 is an unrooted neighbor-joining tree showing the genetic relationships among diplogonoporiid isolates estimated using *cox1* (A) and ITS-1 region (B) sequences. The transitional (A ⇄ G, T ⇄ C) and transversal substitutions (T ⇄ G, T ⇄ A) were at 25 and 2 sites (1393 and 1476), respectively, along the 1566-bp *cox1* sequence (data not shown). The genetic distance ranged from 0.0000 to

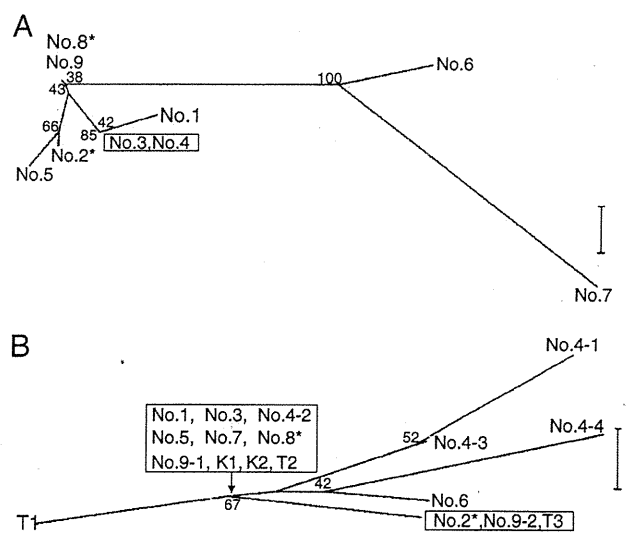

Fig. 1. Unrooted neighbor-joining trees inferred from the sequences of the (A) *cox1* (1566 bp) and (B) ITS-1 region (625 bp) of *Diplogonoporus* isolates. Numbers at the nodes indicate bootstrap values (1000 replicates). Asterisks denote samples used for the complete mitochondrial genome analysis. Bars indicate genetic distances calculated by K2P model. ITS-1 regions of *D. grandis* isolates K1, K2, T1, T2 and T3 are from accession numbers AB355629, AB298510, AB298512, AB298513 and AB298514, respectively. Bars = 0.001 (A) and 0.0005 (B).

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0.0158 (overall mean = 0.0059), which are typical of intraspecific variations. Nucleotide sequences of the ITS-1 region (625 bp) of 2 *Diplogonoporus* isolates (Nos. 4 and 9) were determined using PCR products subcloned into pT7 Blue T-vector (Novagen, USA) because dual peaks at 4 sites were detected by direct DNA sequencing. Polymorphic nucleotides were observed at 4 sites (30, 147, 334 and 548) in *D. balaenopterae* (No. 4) and at position 344 in *D. grandis* (No. 9) (data not shown). Transitions (A ⇄ G, T ⇄ C) and transversions (C ⇄ A) were observed at sites 344 and 548, and 30 and 147, respectively. The genetic distance values ranged from 0.0000 to 0.0048 and overall mean was 0.0017, which are also typical of intraspecific variation. Thus, based on mitochondrial and nuclear DNA data, the present study confirmed that *D. grandis* is a junior synonym of *D. balaenopterae* and thus, causative agent of human diplogonoporiasis should be called *D. balaenopterae*.

3.3. Taxonomic and phylogenetic relationships between the genera *Diplogonoporus* and *Diphyllobothrium*

The Pseudophyllidea van Beneden in Carus, 1863, a well-recognized order of tapeworms (Platyhelminthes: Eucestoda), has been considered to be a monophyletic group of difossate cestodes [40]. However, phylogenetic analysis based on nuclear ribosomal RNA genes and ITS-2 sequences revealed the presence of two phylogenetically unrelated groups, indicating paraphyly or polyphyly of the order Pseudophyllidea [15,17,19,20]; consequently, two new orders, Diphyllobothriidea and Bothriocephalidea, have been proposed to accommodate these unrelated lineages [41]. In addition another study [24], the mitochondrial DNA results of this study also support the proposal. However, the taxonomic and phylogenetic relationships between the genera *Diplogonoporus* and *Diphyllobothrium* (Diphyllobothriidae, Diphyllobothriidea) have been not yet been clarified in sufficient detail, primarily because DNA sequence data are only available for a limited number species [42], and also because the taxonomic positions of several species within these genera are still uncertain.

Fig. 2 shows phylogenetic trees inferred by ML and BI algorithms using nucleotide sequences of the *cox1* (356 bp). Although two tree topologies were somewhat different, *Diplogonoporus* isolates formed monophyletic clade belonging to Diphyllobothriidae (Diphyllobothriidea) with strong nodal support (≥75 in ML, ≥0.97 in BI) and were more closely related to *D. stemmacephalum*, the type-species of the genus *Diphyllobothrium*, corroborating the findings of a previous study [24]. Phylogenetic studies on pseudophyllidean (= diphyllobothriidean and bothriocephalidean) cestodes support paraphyly and/or polyphyly of several cestode genera, including *Ligula* Bloch, 1782 and *Bothriocephalus* Rudolphi, 1808 [17,19,22], and *Diphyllobothrium*[43]. The close relationship between *Diplogonoporus* spp. and *D. stemmacephalum* implies that these taxa constitute a paraphyletic group.

Interestingly, *D. balaenopterae* and *D. stemmacephalum* are phylogenetically closely related and infect cetaceans, such as *Balaenoptera* spp. and *M. novaeangliae* (Balaenopteridae) [1,2], and the harbor porpoise (*Phocoena phocoena*, Phocoenidae, Cetacea), bottlenose dolphin (*Tursiops truncatus*, Delphinidae, Cetacea) and long-finned pilot whale (*Globicephala melas*, Delphinidae, Cetacea) [1], respectively. Based on these similarities in host preference, it seems likely that *D. balaenopterae* and *D. stemmacephalum* are derived from a common ancestral species [24].

The genus *Diplogonoporus* has been characterized as having two sets of genitalia in a single proglottid [1]. However, additional genitalia (3 to 5 pairs) have been observed in some segments of *D. balaenopterae* collected from an Antarctic sei whale (*B. borealis*, Balaenopteridae, Cetacea) [44]. Similarly, in dwarf forms of *D. balaenopterae* (body length: 61–809 mm, max. width 2.0–5.6 mm) obtained from a minke whale, although two sets of genitalia per segment were usually encountered, sometimes 4 paired genitalia were observed in a single proglottid in the same individuals [45]. Conversely, two sets of reproductive organs

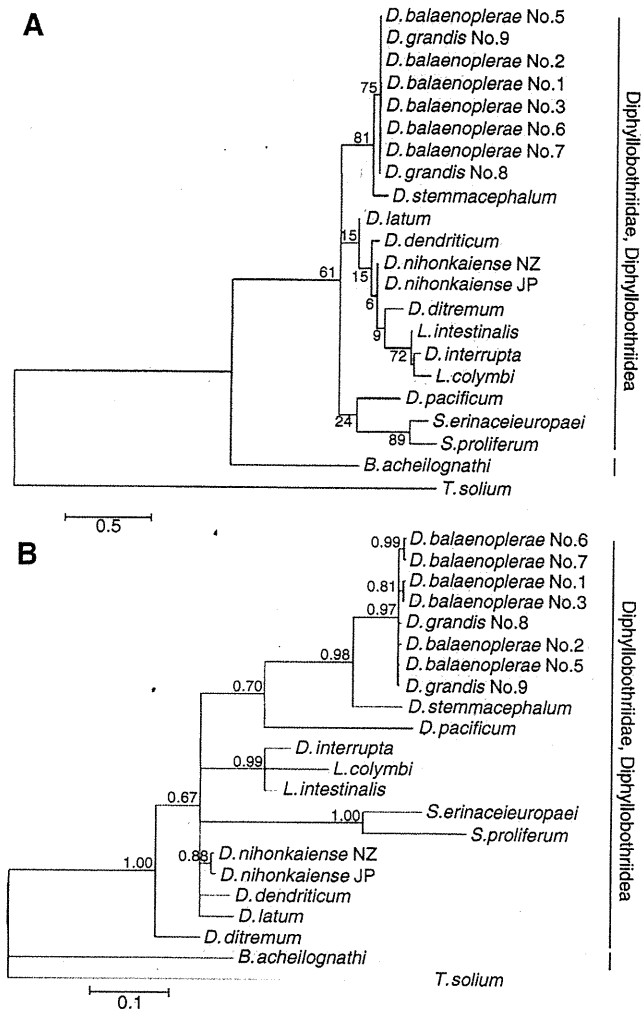

Fig. 2. Phylogenetic trees constructed by the (A) maximum likelihood and (B) Bayesian inference analyses using *cox1* sequence data (356 bp). Nucleotide sequence data were obtained from the following accession numbers: *Diplogonoporus balaenopterae* (AB355622–AB355624, AB355626, AB474567, AB474568), *Diplogonoporus grandis* (AB355628, AB355629), *Diphyllobothrium stemmacephalum* (DQ768191), *Diphyllobothrium pacificum* (AB517949), *Spirometra erinaceieuropaei* (AB369249), *Sparganum proliferum* (AB015753), *Digramma interrupta* (EU241311), *Ligula intestinalis* (EU241317), *Ligula colymbi* (EU241308), *Diphyllobothrium ditremum* (FM209182), *Diphyllobothrium latum* (AB510496), *Diphyllobothrium dendriticum* (AM412738), *Diphyllobothrium nihonkaiense* (AB268585, AB364645) and *Bothriocephalus acheilognathi* (Bothriocephalidae, Bothriocephalidea, HM439384). *Taenia solium* (Taeniidae, Cyclophyllidea, AB516957) was used as the outgroup. Bootstrap values (1000 replicates) in ML and posterior probabilities in the BI are shown at the branch. Bars indicate the number of base substitutions/site.

per segment have been reported in *Diphyllobothrium yonagoense*, which infects Risso's dolphin (*Grampus griseus*, Delphinidae, Cetacea) [46] and rarely, duplicated genitalia have been reported in *Bothriocephalus*, *Triaenophorus*, *Echinophallus* and *Paraechinophalus* (Bothriocephalidea) [41]. Moreover, in some genera of Diphyllobothriidae (e.g., *Baylisia*, *Tetragonoporus* and *Hexagonoporus*), multiple genital organs have been observed in each segment. Thus, because the number of genitalia can vary, even within species, two sets of genitalia as a taxonomic character may not be sufficiently robust for resolving affiliations among the members of the genus *Diplogonoporus*. In addition, these observations suggest that multiplication of the reproductive organs may be an adaptive phenomenon in cestode evolution.

In so far as the taxonomic placement of *Diphyllobothrium pacificum*, a parasite of the South American sea lion (*Otaria flavescens*,

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Otariidae, Carnivora), is concerned, it has been proposed that *Adenocephalus pacificus* Nybelin, 1931, the original name assigned to the species, should be restored as the valid name based on molecular and morphological data which *D. pacificum* is phylogenetically distant from *Diphyllobothrium* species (i.e., *D. nihonkaiense*, *D. latum*, and *D. dendriticum* etc) [23]. Our results also support the validity of the original genus *Adenocephalus* although the position of *D. pacificum* was different in two phylogenetic trees. Because *Diphyllobothrium* including *D. nihonkaiense*, *D. latum*, *D. dendriticum* and *D. ditremum*, which are all non-cetacean *Diphyllobothrium* parasites, is placed at markedly distant position from *D. stemmacephalum* (Fig. 2). The genera *Digramma* Cholodkovsky, 1914 and *Ligula*, formed a monophyletic cluster, but *Digramma* is considered a synonym of *Ligula*[22,47]. Indeed, *Digramma* may merely be a rare diplogonadic type of *Ligula*.

Regarding the validity of the genera *Diplogonoporus* and *Diphyllobothrium* based on molecular evidence, the following scenarios are possible;

i) *Diplogonoporus* should be synonymized with *Diphyllobothrium*, and the name *Diphyllobothrium* should be used for *D. stemmacephalum*, which may also include several other cetacean *Diphyllobothrium* species
ii) *Diplogonoporus* should be retained as a valid genus
iii) Non-cetacean *Diphyllobothrium* species, including *D. nihonkaiense*, *D. latum* and *D. dendriticum*, should be placed in a newly designated genus.

To conclude whether *Diplogonoporus* can be regarded as a synonym of *Diphyllobothrium* or not, comprehensive molecular-phylogenetic analyses should be undertaken using other *Dipologonoporus* species, such as *Diplogonoporus tetrapterus*, which infects the harbor seal (*Phoca vitulina*, Phocidae, Carnivora) and fur seal (*Callorhinus ursinus*, Otariidae, Carnivora), and *Diplogonoporus violettae* which infects the sea lion (*Eumetopias jubatus*, Otariidae, Carnivora). In addition, the gigantic and dwarf forms of *D. balaenopterae*[45,46], as well as species that infect other cetacean species, including *D. yonagoense* and *Diphyllobothrium macroovatum* which infect minke whales (*B. acutorostrata*) and gray whale (*Eschrichtius gibbosus*, Eschrichtiidae, Cetacea) [48] should be also examined. Moreover, other genera related to *Diplogonoporus*, including *Tetragonoporus*, *Hexagonoporus* and *Polygonoporus*, are required in order to clarify the phylogenetic relationships among these cestodes, and molecular analyses using *D. yonagoense* and *Hexagonoporus* isolates are currently underway. For scenario iii), the genus *Diphyllobothrium* should contain the type-species of *D. stemmacephalum*. It may therefore be reasonable to place the non-cetacean *Diphyllobothrium* species into a new genus that is distinct from *Diphyllobothrium*. To revise the validity of the genus *Diphyllobothrium*, further molecular analysis using more non-cetacean *Diphyllobothrium* taxa would be necessary.

In conclusion, the findings of the mitochondrial and nuclear DNA analyses reported here will be very useful, not only for analyzing the phylogenetic relationships among eucestodes, but also for differentiating *Diplogonoporus* species from *Diphyllobothrium* species. In addition, larval stages of *D. balaenopterae* have not yet been discovered. It will now be possible to determine whether plerocercoids found in marine fish hosts are *D. balaenopterae* or not, which will further clarify the life cycle of this parasite and facilitate the prevention of diplogonoporiasis in humans.

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Molecular identification of a causative parasite species using formalin-fixed paraffin embedded (FFPE) tissues of a complicated human pulmonary sparganosis case without decisive clinical diagnosis ☆

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ABSTRACT

PCR-based molecular diagnosis was made for the identification of causative agents of the clinically suspected pulmonary proliferative sparganosis case found in Thailand using formalin-fixed paraffin-embedded (FFPE) biopsy specimens. As a reference, FFPE biopsy specimen from a typical cutaneous sparganosis case was examined together. DNA samples were extracted from tissues and two partial fragments of cytochrome *c* oxidase subunit 1 (*cox1*) gene were amplified for the detection of *Spirometra* DNA. Two *cox1* fragments were amplified successfully for both specimens. After alignment of nucleotide sequences of the PCR-amplicons, the causative agents of both cases were identified as *Spirometra erinaceieuropaei*.

1. Introduction

Human sparganosis is a zoonosis caused by plerocercoid larva called sparganum of pseudophyllidean (now classified as diphyllobothriidean [1,2]) tapeworm [3,4]. The disease is classified into two forms, non-proliferative sparganosis and proliferative sparganosis. The former is caused by infection with canine and feline tapeworm, genus *Spirometra*, whereas the latter is caused by infection with *Sparganum proliferum*, of which adult worm remains unknown [3,4]. Genus *Spirometra* exploits freshwater copepods as the first intermediate host and an array of amphibians, reptiles, birds and mammals as the second intermediate hosts/paratenic hosts depending on the food chain [3,4]. Human non-proliferative sparganosis is endemic mainly in East and Southeast Asia, especially in China, Japan, Korea, Taiwan and Thailand [5–9]. Sporadic cases were reported also from the Americas [10]. The causative agents for sparganosis in Asia is *Spirometra erinaceieuropaei*, whereas in the Americas is *Spirometra mansonoides* [3,4]. Human infection occurs by eating raw or undercooked meat of the second intermediate hosts or paratenic hosts, especially frogs and snakes. In addition, infection occurs by drinking water contaminated with copepods, or by the usage of frog meat poultice [3,4]. The disease is characterized by migrating tumor and granulomatous lesion surrounding the worm preferentially in the subcutaneous tissues and less frequently in the eyes and central nervous system (CNS) [5–9]. In contrast to typical non-proliferative sparganosis, proliferative sparganosis caused by infection with *S. proliferum*, of which adult worm is not yet known [3,4], is extremely rare but clinically aberrant with high mortality. Until now, a total of 16 cases have been reported sporadically from various countries (6 cases in Japan, 3 in Taiwan, 2 each in USA and Thailand, and one each in Paraguay, Venezuela and Reunion Island) (see Supplementary Table). Recent molecular analyses on mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear succinate dehydrogenase iron–sulfur protein subunit (*sdhB*) genes revealed that *S. proliferum* is similar but distinct from *S. erinaceiuropaei* [11,12]. In Thailand, 54 sparganosis cases, 52 non-proliferative and 2 proliferative sparganosis, were

☆ Nucleotide sequence data reported in the present paper are deposited in the DDBJ/GenBank/EMBL databases under the accession numbers AB612881 and AB612882 the regions of 650–800 and 965–1120 of the cytochrome *c* oxidase subunit 1 gene, respectively.

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identified by the literature survey [9]. Among 52 non-proliferative cases, one case of pulmonary sparganosis [13,14] is extremely difficult to identify whether this is proliferative or non-proliferative sparganosis [9], because the patient had multiple lesions in both lungs by spargana with the progress of the disease and the eventual death by *Cor pulmonale*[9,15]. Here we report the molecular evidence that this unusual pulmonary sparganosis case was *S. erinaceiuropaei* infection using the paraffin block preserved for over 15 years.

2. Materials and methods

2.1. Patients and the specimens

In the present study, formalin-fixed paraffin embedded (FFPE) parasites derived from two Thai male patients with sparganosis were used for molecular diagnosis. The paraffin block had been preserved in the Department of Pathology, Faculty of Medicine, Khon Kaen University, Thailand. Ethics approval for the study protocol was received from the Khon Kaen University Ethics Committee for Human Research (HE 541086).

Patient #1 is a 65 year-old man from Udon Thani Province, northeast Thailand, with subcutaneous abdominal sparganosis which is supposed to be *S. erinaceiuropaei*. He received simple surgical resection of the nodular lesion for diagnostic treatment on May 2006. Postoperative histopathological diagnosis confirmed that this case was a non-proliferative sparganosis and the residual biopsy specimen (code #, S49-3754) has been kept in the Department of Pathology, Faculty of Medicine, Khon Kaen University. Patient #2 is a 37 year-old also from Udon Thani province, northeast Thailand. He was misdiagnosed as pulmonary tuberculosis because the chest radiography revealed diffuse multiple nodular infiltrations with cavitations, and had been treated with antituberculosis drugs for one year without improvement. Eventually this patient had received an open lung biopsy in 1995. Pathological section revealed the presence of spargana in the specimen (code #, S37-61; [13]). After 5 year follow-up, his bilateral multifocal lung lesions rather diversified and he died of *Cor pulmonale* [14]. Because of the clinical features and histopathological findings, this case was suspected of *S. proliferum* infection [9,15].

2.2. DNA extraction, polymerase chain reaction, DNA sequencing and sequence analysis

To extract DNA efficiently from FFPE specimens, thin sections (10 μm-thickness) were prepared and the parasites in the sections were confirmed by hematoxylin–eosin stain prior to preparing DNA (Fig. 1). DNA was extracted from unstained serial sections attached on the glass slides using a DEXPAT kit (TaKaRa Bio Inc., Japan) as reported previously [16,17]. Briefly, the resulting supernatants were used as DNA template for polymerase chain reaction (PCR). Amplification of mitochondrial *cox1* gene by PCR was performed in a 25-μL reaction mixture. KOD FX DNA polymerase (TOYOBO, Japan), which is a DNA polymerase derived from the hyperthermophilic Archaeon *Thermococcus kodakaraensis* KOD1 and with high fidelity was used. We chose *cox1* gene as the target DNA because this has been used for molecular phylogenetic study of *S. proliferum* among pseudophyllidean tapeworms, and was proven as the suitable marker to distinguish *S. proliferum* from *S. erinaceieuropaei*[11]. Assuming that DNA has been degraded by formalin fixation, to amplify short DNA fragments, the primer pairs were designed using the Primer3 software [18] based on *cox1* sequences of *S. erinaceieuropaei* and *S. proliferum* as follows: F650 (5′-CGG CTT TTT TTG ATC CTT TGG GTG G-3′) and R800 (5′-GTA TCA TAT GAA CAA CCT AAT TTA C-3′), and F965 (5′-CTT GGC TTT ATA TGA TTT AAA TAG T-3′) and R1120 (5′-CAA ACC ACG TGT CAT GCA AAA TTT T-3′) for *S. erinaceieuropaei*; F259 (5′-CCT CTA TTA TCG GGC CTT CC-3′) or F366 (5′-GGC TGG GAT AGG GTG AAC TT-3′) and R472 (5′-TTG AAA CCC CAG CTA AAT GC-3′), F453 (5′-GCA TTT AGC TGG GGT TTC AA-3′) and R684 (5′-AGG ATC TCC ACC CCC TAA TG-3′) and F462 (5′-TGG GGT TTC AAG TTT GTT GG-3′) and R611 (5′-GCC GCT AAT ACC GGA ATA GTT-3′) for *S. proliferum*. The numbers of primers correspond to positions of the *cox1* genes of *S. erinaceieuropaei* and *S. proliferum* consisting of 1566 bp and 1496 bp, respectively. Although nested-PCR using outer-universal and inner-specific primer sets is the standard approach for this kind of research, we employed repeated PCR using the same primer and the same cycle condition because the target gene fragments were too short to design outer and inner primers. The PCR was performed at 94 °C for 15 min followed by 35 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 30 s) and extension (72 °C, 60 s) plus 1 cycle of 72 °C for 5 min. After first PCR reaction, for *S. erinaceieuropaei* amplification, the amplified product was added to the second reaction mixture for re-amplification by using the same pair of primer sets and the same condition as above. For the first PCR amplified product by using *S. proliferum* specific primer pair (F259/R472), the F259/R472 and F366/R472 primer pairs were used for re-amplification and semi-nested PCR, respectively, whereas the amplification by using F453/R684 primer pair, the same and F462/R611 primer pairs were used for re-amplification and nested PCR, respectively.

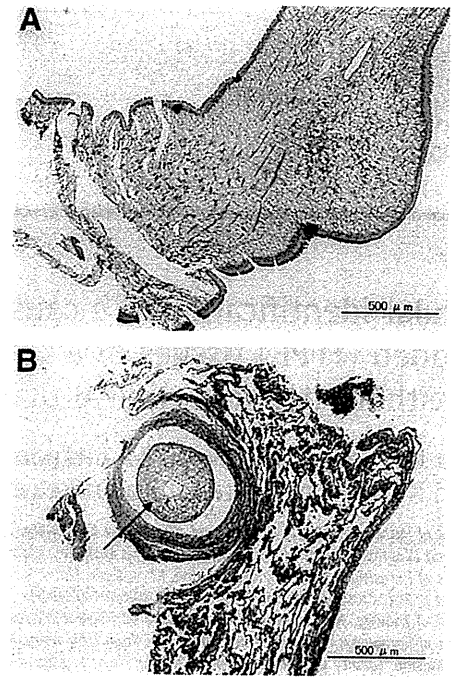

Fig. 1. Plerocercoids detected in the paraffin-embedded sections used for molecular identification. A. A plerocercoid isolated from the subcutaneous nodule of patient #1; B. A plerocercoid (arrow) detected in the lung tissue from patient #2. The sections were stained with hematoxylin–eosin.

Amplicons were confirmed by 2–3% agarose gel electrophoresis and purified using NucleoSpin Extract II kit (Macherey-Nagel, Germany) for direct DNA sequencing. Samples for the sequencing were prepared using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., CA) and sequencing was performed on 3100 Genetic Analyzer and/or 3730 xl DNA Analyzer (Applied Biosystems Inc., CA). Sequence analysis was performed using Clustal W software [19].

3. Results

In the molecular analysis of the plerocercoids from case #1 (Fig. 2A), the target DNAs were re-amplified by the 2nd PCR using two primer pairs (F650/R800 and F965/R1120) specific for the *cox1* gene

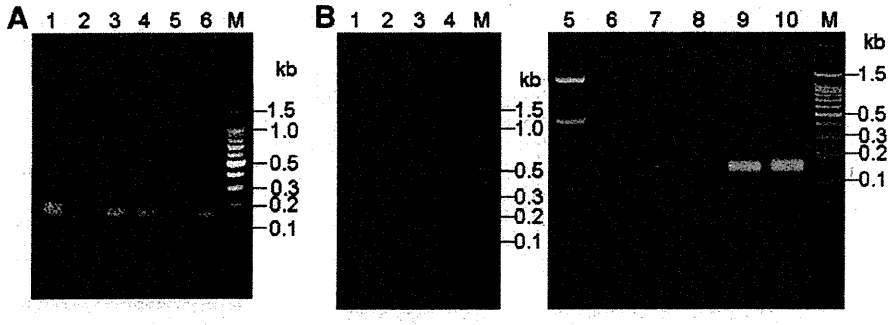

Fig. 2. *Cox1* gene fragments amplified by PCR. A. Patient #1: Lanes 1 and 4, 1st PCR products using *S. erinaceieuropaei* DNA and primer pairs of F650/R800 (151 bp, lane 1) and F965/R1120 (156 bp, lane 4) specific for *S. erinaceieuropaei*. Lanes 2 and 3, the 1st and 2nd PCR products using primer pair of F650/R800. Lanes 5 and 6, the 1st and 2nd PCR products using primer pair of F965/R1120. B. Patient #2: Lanes 1 and 2, the 1st PCR products using primers specific for *S. proliferum* (F259/R472 and F453/R684), respectively. Lanes 3 and 4, the 1st PCR products using primers specific for *S. erinaceieuropaei* (F650/R800 and F965/R1120), respectively. Lanes 5 and 6, the 2nd PCR products using 1st PCR product from lane 1, and primers specific for *S. proliferum* (F259/R472 and F366/R472), respectively. Lanes 7 and 8, 2nd PCR products using 1st PCR product from lane 2, and primers specific for *S. proliferum* (F453/R684 and F462/R611), respectively. Lanes 9 and 10, the 2nd PCR products using 1st PCR products from lanes 3 and 4, and primers specific for *S. erinaceieuropaei* (F650/R800 and F965/R1120), respectively.

of *S. erinaceieuropaei* (151 bp in lane 3 and 156 bp in lane 6 in Fig. 2A, respectively), but not by the 1st PCR (F650/R800 and F965/R1120 in lanes 2 and 5 in Fig. 2A, respectively). In the plerocercoids from case #2 (Fig. 2B), the etiologic agent was supposed to be *S. proliferum*, so that primer pairs specific for the *cox1* genes of both *S. proliferum* and *S. erinaceieuropaei* were tested. As results, neither product was re-amplified by the 2nd PCR using primer pairs specific for *S. proliferum* (F259/R472 and F453/R684 in lanes 5 and 7 in Fig. 2B, respectively) nor product was semi-nested or nested PCR using F366/R472 or F462/R611 primer pairs, respectively (lanes 6 and 8 in Fig. 2B). However target DNAs were re-amplified by the 2nd PCR using primer pairs specific for *S. erinaceieuroapei* (151 bp and 156 bp in lanes 9 and 10 in Fig. 2B, respectively). Homology search of the PCR-amplified products was conducted against regions of 650–800 and 965–1120 of the *cox1* genes of the *S. erinaceieuropaei* reference sequences (AB369249–AB369251, AB015754 and AB374543), and against the same regions of the *S. proliferum* reference sequence (AB015753). The results were summarized and shown in Table 1 and Fig. 3. Obviously both worms from patient #1 and #2 showed high sequence similarities (≥97%) with the two regions of *cox1* sequences of *S. erinaceieuropaei*, but relatively low homologies (≤90%) with those of *S. proliferum*. Then, the plerocercoids obtained from two sparganosis patients from northeast Thailand were both identified as *S. erinaceieuropaei*.

4. Discussion

PCR-based molecular technique is a powerful tool for identification/speciation of causative pathogens of infectious diseases. Genetic information of pathogens obtained from FFPE tissues kept for long years is extremely useful for the retrospective re-appraisal of individual cases and also for epidemiological studies on infectious as well as all other diseases [20]. DNA extraction from FFPE tissues is generally problematic because formalin fixation causes DNA fragmentation and paraffin interferes DNA extraction. However, recent improvement of DNA extraction kits/methods and the development of appropriate primers to detect short fragments allowed us to identify various helminth parasites in FFPE tissues [16,17,21–25]. Our results also clearly showed the usefulness of the application of molecular diagnosis using old FFPE tissues.

In the present study, two short fragments of *cox1* gene were selected as the targets for PCR amplification, because they were already proven as the suitable markers for differentiate *S. erinaceieuropaei* and *S. proliferum*, and also useful for the phylogenetic analysis of pseudophyllidean (diphyllobothriidean tapeworms) [11]. Because the target gene fragments were short, we have repeated the PCR twice using the same primer sets under the same condition, instead of semi-nested and nested PCR using common outer and specific inner primers. Since sufficient amount of PCR amplicons was obtained by the repeated PCR in this study, this repeated PCR method can be applied for the amplification of short DNA fragments from FFPE tissues in general.

For the proliferative sparganosis, a total of 16 cases have been reported sporadically worldwide (see Supplementary Table). Most of the cases have multiple lesions, but lung involvement is extremely rare. Patient #2 in the present study was suspected of having proliferative sparganosis by open lung biopsy followed by autopsy [14], but the molecular diagnosis using specific primer sets revealed that this case was an infection with *S. erinaceieuropaei*. Until now, only two proliferative sparganosis cases, if the laboratory strain isolated from patient was included, have been diagnosed by molecular methods [11]. Whether some of the previously reported proliferative sparganosis cases with limited lesions were really infected with *S. prolifera* need molecular confirmation. Also, whether *S. erinaceieuropaei* turned into proliferative form or not still remains to be solved by molecular examinations of more cases of proliferative sparganosis. Whatever would be the solution, the present study clearly demonstrated the usefulness of molecular diagnosis for visceral sparganosis cases with multiple lesions.

In Thailand, a total of 52 cases of non-proliferative and 2 cases of proliferative sparganosis have been reported for over 50 years [9]. Patient #1 has been reported first time in the present study so that this should be the 53rd case of non-proliferative sparganosis due to *S. erinaceieuropaei* infection in Thailand. Patient #2 of the present study was already listed as the non-proliferative sparganosis with deserved official diagnosis [9,15]. The molecular diagnosis of this study confirmed that previously reported pulmonary sparganosis case is in fact non-proliferative sparganosis due to *S. erinaceieuropaei* infection. The present study also is the first molecular evidence of *S. erinaceieuropaei* plerocercoids from two sparganosis patients (one each of subcutaneous and pulmonary sparganosis) in Thailand.

The majority of non-proliferative sparganosis is caused by a single worm infection. Although infection with multiple worms has been

Table 1
The sequence homology of the PCR-amplified *cox1* fragments against *Spirometra erinaceieuropaei* and *S. proliferum*.

Worms from	*Cox1* sequence homology against			
	S. erinaceieuropaei		*S. proliferum*	
	650–800	965–1120	650–800	965–1120
Case #1	97.0%	98.1%	89.0%	86.7%
Case #2	99.0%	100%	90.0%	87.6%

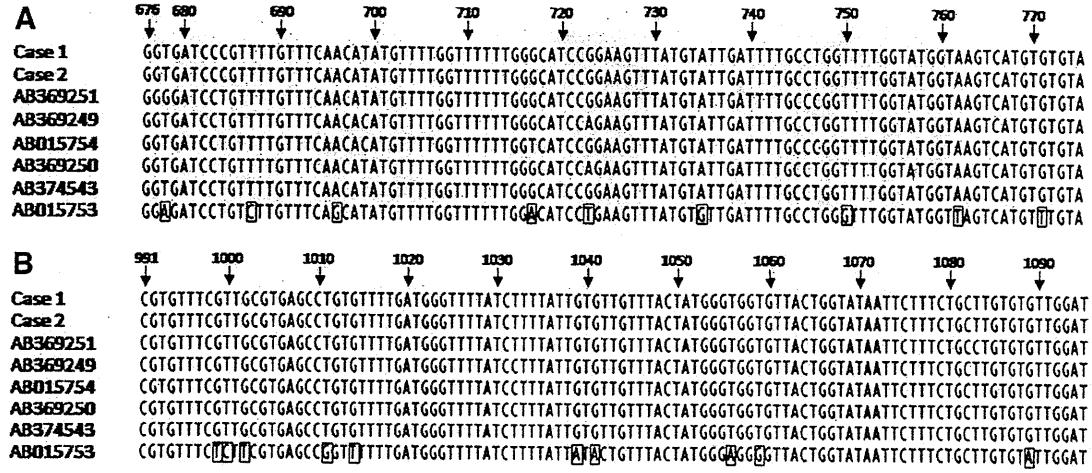

Fig. 3. Alignment of the nucleotide sequences of the PCR-amplified *cox1* gene fragments. A. PCR product (151 bp) using the primer pair of F650/R800. B. PCR product (156 bp) using the primer pair of F965/R1120. Nucleotide sequence data were obtained from the following accession numbers: *S. erinaceieuropaei* (AB369251, AB369249, AB015754, AB369250 and AB374543) and *S. proliferum* (AB015753). Boxed nucleotides indicate nucleotides unique for the *cox1* gene of *S. proliferum*.

sporadically reported, most of them were found as multiple subcutaneous nodular lesions [26]. In Thailand, 3 worms were found from the eyelids of a patient [27]. Recently 5 worms were surgically removed (3 worms being extirpated while she was in Bangkok and 2 worms while she was in Japan) from a Thai-Japanese woman [28]. In this case, the patient applied frog meat as the poultice. In the present study, we were unable to count the exact number of worms in the lungs of patient #2, but definitely more than two worms should present simultaneously because the patient suffered from bilateral lung lesions and the presence of worms in both lungs was confirmed by biopsy [14]. There are a few pleuropulmonary sparganosis cases reported in Taiwan [29], Korea [30] and Japan [31]. However, most of those cases were the infection in the pleural cavity. The involvement of lung parenchyma is extremely rare [30,31]. In Thailand, apart from the present case, only one case of pulmonary sparganosis in that the worm was found in the mediastinal lymph node, was reported [32].

In conclusion, present results clearly show the usefulness of PCR-based identification of causative agents preserved as FFPE tissues for long years.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.parint.2011.07.018.

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マス生食によると思われた日本海裂頭条虫症の1例

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要　旨

日本海裂頭条虫は成虫となるとヒトの消化管内で数mにも及ぶが，排便時等の虫体排泄以外に自覚症状のない例も多い．本例は虫体排泄を主訴に受診され，ガストログラフィンおよびプラジカンテルを用いて駆虫を行った．cox1遺伝子解析より日本海裂頭条虫と虫種同定された．近年，輸送機関の発達により鮮魚類の生食機会が増加してきており，本疾患をはじめとした寄生虫感染は過去のものではないと再認識する必要性が考えられた．

〔日内会誌　100：3336～3338，2011〕

Key words　寄生虫，日本海裂頭条虫，プラジカンテル，ガストログラフィン

症　例

患者：33歳，男性．主訴：虫体排泄．既往歴：胃潰瘍．職業：調理師．現病歴：2009年頃マスの生食歴あり．以降間欠的な腹痛，下痢が続いていた．2010年2月排便時に虫体の排泄を認めた．虫体を持参して近医を受診したところ条虫症の診断を受け，加療のため紹介入院となった．入院時現症：特記すべき所見なし．入院時検査所見：血液検査では血算，生化学に明らかな異常値なし．便虫卵および虫体検査は陰性．

臨床経過

便虫卵検査は計2回陰性であった．入院3日目に挿入した十二指腸ゾンデよりガストログラフィンを初回150 ml，以降10分ごとに50 ml投与し，計500 mlの小腸注入を行ったが，頭節を認めず完全な虫体排泄には至らなかった．このため入院6日目にプラジカンテル1,650 mgを内服させ，その2時間後にクエン酸マグネシウム34 gを内服させたところ，虫体の完全な排泄を得た（図a）．排泄された虫体は1匹で体長約2 m，最大幅約10 mmで，約1 mmの棍棒状の

〔第212回東海地方会（2010/10/09）推薦〕〔受稿2011/04/21，採用2011/06/27〕

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Case Report; A case of *Diphyllobothrium nihonkaiense* infection probably caused by eating raw trout.

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